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Immunochemical Techniques in Biological Monitoring

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Immunoassays are analytical methods that detect interactions between antibodies and antigens. Immunoassays were used originally to detect large biological molecules. The new generation of these antibody-based assays can detect small synthetic compounds. As a result, immunoassays are being developed specifically for biomarkers of exposure and effect to environmentally prevalent chemicals. Immunochemical detection of parent compounds in blood and tissues, metabolites in excreta, and adducts with DNA and protein have been successfully performed by several investigators. Although there is great potential for use of immunoassays in biological monitoring studies, the limitations of these analyses must be fully understood to prevent improper evaluation of the acquired data. This review will cover some of the background material necessary to understand how an antibody-based assay is developed. The differences between polyclonal and monoclonal antibody-based assays and the importance of antibody class, affinity, specificity, and cross-reactivity must be considered in both study design and data analysis.

Biological Monitoring

Biological monitoring is used to measure the absorbed dose of a chemical following an exposure. It is an important measurement since only the internalized chemical is bioavailable to interact with the biological system. The monitoring measures either a pharmacokinetic or pharmacodynamic variable. Pharmacokinetic variables are biomarkers of exposure. The pathological change caused by a chemical on the biological system is a pharmacodynamic effect. Most often monitoring has measured pharmacokinetic variables such as the concentrations of the parent chemical or its metabolites in blood, tissue, expired breath, and excretory levels following an exposure. Most pharmacodynamic variables are biomarkers of effect. For example, decrease in blood pseudocholinesterase activity following exposure to organophosphate pesticides is a pharmacodynamic effect.

Some chemical compounds or their metabolites are capable of covalently binding to biological molecules such as DNA and proteins to form adducts in target and peripheral tissues. Many of these adduct-forming compounds have also been shown to be carcinogenic. Although the measurement of adducts in peripheral tissues has often been found to be proportional to external exposure, no correlation between the adduction and tissue-specific cancers has ever been proven. The measurement of blood component adducts (hemoglobin, albumin, and lymphocyte DNA) as a method of biological monitoring is currently being investigated (Table 1) since these adducts are important as biomarkers of exposure. Immunoassays are well suited to detect biomarkers of exposure.

Immunoassays

"Immunoassay" is a generic term for any method that detects the interaction between an antibody and an antigenic analyte. This interaction can be used to identify, localize, purify, and quantitate the analyte. Originally, immunoassays were developed to detect macromolecular aggregates, such as bacteria and viruses. Detection of small molecular weight compounds (drugs,

Table 1. Immunoassays in biomonitoring.

Exposure	Biomarker[a]	Location	Year	Reference
Coke ovens	BaP-DNA	Netherlands	1990	(*16*)
Roofing	BaP-DNA	U.S.	1985	(*12*)
Firefighters	BaP-DNA	U.S.	1989	(*14*)
Barbecue meat	BaP-DNA	U.S.	1989	(*14*)
Dietary	NNO-DNA	China	1986	(*4*)
Dietary	AFB_1-SA	China	1988	(*11*)
	AFB_1 in urine	Philipines	1986	(*4*)
DDT	DDA in urine	India	1987	(*5*)
Cigarette smoke	ORP in urine	U.S.	1985	(*6*)
	ORP in serum	U.S.	1988	(*7*)
Ethylene oxide	2-hydroxyethyl-Hb	Europe	1988	(*10*)
Coke ovens	anti-BaP-DNA Ab	U.S.	1985	(*8*)
Phthalic anhydride	anti-PH-SA Ab	U.S.	1987	(*9*)

[a]BaP, benzo[*a*]pyrene adducts; NNO-DNA, *N*-nitrosamine-DNA adducts; AFB_1-SA, aflatoxin B_1-serum albumin adducts; AFB_1, aflatoxin B_1; DDA, 1,1,1-trichloro-2,2-*bis*(*p*-chlorophenyl)acetic acid; ORP, oncogene-related protein; 2-hydroxyethyl-Hb, hydroxyethyl-hemoglobin adducts; anti-BaP-DNA Ab, anti-benzo[*a*]pyrene-DNA adduct antibodies in serum; anti-PH-SA Ab, anti-phthalic anhydride-serum albumin adduct antibodies in serum.

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industrial chemicals, etc.) by immunoassay has been used for about 35 years and was initiated in 1917 (*1*). Immunoassays are now being developed to detect environmental contaminants found in the excreta and tissues as parent chemical, metabolites, and adducts (Table 1). The major advantages of the immunoassay method for performing this environmental biomonitoring are sensitivity, specificity for the analyte, simplicity and speed of analyses for multiple samples, and ease of standardization.

Immunochemistry Definitions

Antigens are substances that are recognized by the immune system as foreign to the host and induce an immune response. An immune response may or may not involve the production of soluble antibodies (some immune responses are cell mediated). The part of the antigen to which the antibody binds is the antigenic determinant or epitope. Antigens have many different potential determinants, such as various portions of a bacterial cell wall.

Antibodies are a class of proteins that are induced following introduction of an antigen. Because these proteins are found in the gamma globulin fraction of serum, the term "immunoglobulins" (Ig) is synonymous for antibodies. There are five main classes of antibodies: IgG, IgM, IgA, IgD, and IgE. IgG is the major class of serum immunoglobulins. IgG antibodies have a molecular weight of about 150,000 and possess four peptide chains: two identical heavy chains (MW 50,000) and two identical light chains (MW 25,000). IgM is the first class of antibody produced during the primary response. It is a pentamer of the basic IgG antibody structure, which itself contains two combining sites, or paratopes. These paratopes are the sites of antibody interaction with the antigen and are of equal affinity and specificity for the determinant of the antigen. This region of the immunoglobulin molecule is called the variable region.

IgG constitutes most of the secondary response to antigens. Because of this, most immunoassays use IgG antibodies. IgM has ten identical variable regions. Immunologists estimate that over 11 million variable regions are possible (*2*). This diversity is exploited in antigen-directed immunoassays by preparing antibodies with the desired paratope.

Haptens are chemicals that can bind to a region of the paratope of an antibody but cannot elicit an antibody response by themselves. Haptens usually have molecular weights less than 1000 (*3*). These small molecules can be coupled to larger molecules (carriers) to yield synthetic antigens. The elicited antibodies may recognize the hapten as part of the epitope.

Antibody Preparation

The process of eliciting, purifying, and characterizing an antibody for an immunoassay is expensive and time consuming. Animals are first immunized with the target antigen or hapten-carrier molecule. If antibody levels (titers) in the sera are high with the appropriate specificity for the antigen, then either the sera or antibody-producing cells are isolated.

Normally an organism has only a small population of antibodies with a given variable region. Following immunization, there is a rise in the serum titer of antibodies that recognize the injected antigen. Antigen-recognizing antibody is elicited as follows. Specialized cells located in the speen, called B-lymphocytes, display antibody on their surface membrane. When antigen binds to the membrane-bound antibody, the B-lymphocyte is stimulated to either divide into identical daughter cells or mature into a plasma cell that is capable of secreting antibody into the serum. The daughter cells follow the same pattern when an antigen binds to their surface antibody. The antibodies produced by a given B-lymphocyte and all its progeny possess identical paratopes and so are monoclonal. Antibodies from different B-lymphocyte parent cells are polyclonal.

Polyclonal antibodies can be prepared quite easily by purifying the sera of immunized animals. This polyclonal sera contains many different antibodies including one or more with affinity for the desired analyte. Functionally, this is often not a problem since one paratope with a high affinity for the antigen will dominate the observed binding characteristics. Monoclonal antibodies offer the possibility of isolating a rare antibody in the polyclonal mixture that binds to a hapten with high affinity. Preparation of monoclonal antibodies is more involved than that of polyclonal antibodies. The B-lymphocytes are isolated from the spleen of an immunized animal. These mortal cells are then chemically fused with an immortal, established myeloma cell line. The resulting hybrid cells (hybridomas) retain the characteristics of producing antibody and immortality. The hybridomas are isolated as individual cells and grown in culture. All the daughter cells will produce monoclonal antibodies that may be isolated from the culture medium or from ascites fluid after injection of the hybridomas into a host animal.

After the antibodies are prepared, they can be used in a wide variety of immunoassay formats. Most schemes have the analyte-directed antibody with a marker or a labeled second antibody that recognizes the analyte-directed antibody. The label or marker provides the basis for quantification.

Development of Immunoassays

There are four major stages in the development of an immunoassay: antibody production, antibody characterization, method validation, and final field studies. This entire process may take several months to years to complete. The requirements of the analyses should determine the need for making a monoclonal or polyclonal antibody. Production of antibodies was previously discussed.

Characterization includes evaluation of the specificity and affinity of the antibody. The class of the antibody should also be determined since polymeric immunoglobins (IgM) can complicate the analyses. The cross-reactivity for similar antigens or chemical structures should be determined and reported. Often, similar chemical compounds will exhibit measurable binding to the same antibody. If these structurally similar compounds are present in test substances, the qualitative and quantitative analysis will be affected. If using an immunoassay prepared commercially or by another investigator, it is critical to fully understand the limitations of the antibody. The final immunoassay can only be as good as the antibody with regard to sensitivity and specificity. Once the antibody has been characterized fully, different formats of immunoassay should be tested for the best specificity, sensitivity, and reproducibility.

Methods validation for the immunoassay requires determination of the linear range for analyses, recovery studies using standards, and correlation with other chemical methods.

Use of Immunoassay to Detect Biomarkers

Several factors must be considered to determine if development or use of an immunoassay is appropriate for a field study. The use of a specific antibody-based assay requires that the identity of the exposure agent(s) is known. Chemical analyses should be employed to measure weakly toxic agents that only require detection at high levels. Compounds that can be easily detected by more classical means rarely warrant immunoassay development unless a less expensive screening method is desired. An immunoassay should not be developed for an agent that will only be studied a few times due to the considerable cost of development.

There are several circumstances that are ideal for using immunoassays. Agents that are a major public health concern that will require repetitive studies justify the expense of resources to develop the antibody. Agents that require low limits of detection that cannot be obtained by classical chemical analyses and samples that require excessive preparation due to matrix interference are ideal situations for immunoassay use. However, one should be cautioned that immunoassays can also be greatly affected by matrix interferences. Immunoassay analyses are recommended for situations that require quick turn around or as a screening test to guide future studies. Antibody-based analyses are becoming more prevalent in places where complicated chemical analyses are not feasible, such as Third World nations (*4,5,11*).

Several biological monitoring studies have been performed by various investigators using immunoassay for detecting biomarkers (Table 1.) In these studies, biomarkers of exposure (urinary metabolites and macromolecular adducts) and biomarkers of effect (oncogene-related proteins) were all measured. An immunoassay capable of detecting aflatoxin B_1 was used to compare the amounts found in the urine of Philippine and French residents (*4*). The Philippine residents had 13 times higher levels than the residents of France. This was probably due to higher levels of contamination in Philippine foodstuffs.

Sometimes it is more appropriate for the immunoassay to detect an important metabolite rather than the parent compound. The lipid-soluble pesticide is not readily excreted from the body. The major urinary metabolite of DDT is the acetic acid derivative of the parent, DDA. An immunoassay was developed in India that can be perfomed on crude urine without extensive sample preparation (*3*).

Immunoassays have been used to detect proteins coded by oncogenes. The function of these protein products is not known, but their presence signals that the oncogenes have been activated. Oncogene activation is usually a result of retroviral infection, but some chemical carcinogens may also turn on these genes (*6*). The oncogene-related proteins were found to be associated with smoking (*7*).

A variation of immunoassay can be used to detect the immunologic response rather than a chemical antigen. The presence of macromolecule adducts can cause the body to mount an antibody response to the altered biomolecules. A high titer of antibodies to the altered biomolecules in the exposed person's serum is evidence of the formation of adducts.

Immunoassays to detect adduction in a wide variety of macromolecules have been successful. Antibodies that recognize benzo[*a*]pyrene-modified DNA have been found in coke oven workers (*8*); antibodies that recognize phthalic anhydride-modified albumin have been detected in chemical production workers (*9*). Hospital workers that use ethylene oxide sterilization have been shown to have increased 2-hydroxyethyl-hemoglobin adduct levels when compared to other hospital employees (*10*). Aflatoxin serum albumin adducts were found to correlate well with urinary metabolites in Chinese people that had a large dietary intake of aflatoxins from their corn consumption (*11*). DNA adducts of *N*-nitrosamines and polycyclic aromatic hydrocarbons have been analyzed by immunoassays. Benzo[*a*]pyrene-DNA adducts were measured in the lymphocytes of roofers (*12*), foundry (*13*) and coke oven workers (*8*), firefighters (*14*), smokers (*15*), and following consumption of barbequed meat (*14*).

Summary

Immunochemical analyses of biomarkers of exposure have been successfully developed by several investigators. Although there is great potential for use of immunoassays in such biological monitoring studies, the limitations of these methods must be fully understood to prevent improper evaluation of the acquired data. The differences between polyclonal and monoclonal antibody-based assays and the importance of antibody class, affinity, specificity, and cross-reactivity must be considered in the study design and the data analysis.

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